DNA VACCINE CONTAINING TUMOR-ASSOCIATED GENE AND CYTOKINE GENE AND METHOD OF PREPARATION THE SAME

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a DNA vaccine and its preparation, which employs DNA recombination technique to co-incorporate one fragment of tumor-associated gene and one fragment of cytokine gene into a vector, thereby producing a DNA vaccine containing one fragment of tumor-associated gene and one fragment of cytokine gene simultaneously.

Description of Related Art

The immunotherapy of cancer is drawing more attention in recent years. In particular along with the development of molecular biology and the advancement of biotechnology, the making of cancer vaccines has seen significant breakthrough. Currently the types of cancer vaccine available include at least DNA vaccine, dendritic cell vaccine and gene-modified tumor vaccine. Unlike a typical vaccine that is used to prevent a disease, cancer vaccine aims at treating cancer. More specifically, the purpose of cancer vaccine is to boost the body immunity to tumor cells and enable the immune system to recognize and kill tumor cells. DNA vaccine introduces gene encoding specific tumor-associated antigen (e.g. oncogene neu, met or ras) into the host cell where said tumor-associated antigen is expressed through the mechanism of transcription and translation to elicit the immune response of the host against said tumor-associated antigen and to achieve the effect of inhibiting or retarding the growth of tumor cells.

In the example of oncogene neu (also called Her-2 or c-erbB-2), previous studies found that neu gene overexpressed in the tumor tissues of some patients with lung cancer, breast cancer, ovarian cancer or bladder cancer. The oncogene neu encodes a transmembrane glycoprotein, which is a

growth factor receptor that can receive message of accelerating cell growth and division. Given the positive correlation between the overexpression of neu gene and propagation of tumor cell, it may be treated as a tumor-associated antigen. In addition, the overexpression of neu gene is related to drug resistance in chemotherapy. Patients with such condition usually have poor prognosis.

Exactly because of its overexpression in certain types of cancer, neu gene may be used to design cancer vaccines that target it, for instance, a DNA vaccine that carries neu gene. The combined use of neu DNA vaccine and cytokine-specific tumor vaccine, such as Interleukin-2 (IL-2), Interleukin-4 (IL-4), and GM-CSF (granulocyte macrophage colony-stimulating factor) has been shown to inhibit the growth of tumor cells in mice. But the preparation of such tumor vaccine requires prolonged culture and screening of tumor cells in vitro. In the culturing process, mutation is prone to occur that results in the loss of surface antigen; in the screening process, the heterogeneity of the tumor cells might be reduced that narrows the protection range of tumor vaccine. Plus the fact that it is costly to prepare this kind of vaccine, its clinical application so far has been limited.

SUMMARY OF THE INVENTION

In addressing the drawbacks of prior arts, the present invention aims to provide an easily prepared and relatively low-cost DNA vaccine and its preparation process. The foregoing DNA vaccine is prepared by incorporating into a vector that contains a suitable promoter or translation regulatory sequence; at least one fragment of tumor-associated gene; and one fragment of cytokine gene. The resulting DNA vaccine contains both tumor-associated gene and cytokine gene.

The expression of tumor-associated gene and cytokine gene incorporated into the aforesaid vector may be controlled by one or more mammalian expression promoters or regulated by IRES (internal ribosome

entry site).

Another objective of the present invention is to provide a method of preparing DNA vaccine containing tumor-associated gene and cytokine gene, comprising at least the following steps: designing a primer sequence containing proper restriction site; using polymerase chain reaction (PCR) to amplify and isolate aforesaid tumor-associated gene and cytokine gene respectively; using ligase to co-incorporate respectively the tumor-associated gene and cytokine gene into a vector having a suitable promoter or translation regulatory sequence.

The aforesaid tumor-associated gene and cytokine gene on the vector may be arranged in such an order that the tumor-associated gene is located in front or behind the cytokine gene.

The aforesaid co-incorporation can be achieved at least by way of: combining the tumor-associated gene and the cytokine gene into a fusion gene to be controlled by the same promoter; or having two independent genes that are controlled respectively by two separate promoters; or having two independent genes that are respectively controlled by a promoter and regulated by an IRES segment.

The aforesaid DNA vaccine may be carried at least by retroviral vector, adenoviral vector, adeno-associated viral vector, or liposome, or administered directly in the form of DNA.

The aforesaid DNA vaccine may be administered at least by way of subcutaneous injection, intramuscular injection, oral administration, spraying or gene gun injection.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a diagram of primer sequence designed in Embodiment 1 according to the present invention.

FIG. 2 is a diagram of N'-neu-IL-2 fusion DNA vaccine in Embodiment 1 according to the present invention.

FIG. 3 is the experimental flow in Embodiments 2, 3 and 4 in accordance with the present invention.

FIG. 4 is a graph showing the tumor-suppressing effect of N'-neu-IL-2 fusion DNA vaccine as depicted in Embodiment 2 according to the present invention.

FIG. 5 depicts the survival rate of mice that received respectively the treatment of normal saline (line a), pRc/CMV vector only (line b), N'-neu DNA vaccine and IL-2 DNA vaccine given separately (line c), N'-neu DNA vaccine (line d) or N'-neu-IL-2 fusion DNA vaccine (line e) as performed in Embodiments 3 and 4 according to the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a DNA vaccine and its preparation, which employs DNA recombination technique to co-incorporate a fragment of tumor-associated gene and a fragment of cytokine gene into a vector. The administration of an effective dose of such DNA vaccine in a mammal may enhance the immune response of the host through the co-expression of tumor-associated antigen and cytokine, and thereby achieve the therapeutic effect of inhibiting or retarding tumor growth.

The main objective of the present invention is to provide a DNA vaccine, which is prepared by incorporating into a vector containing a suitable promoter or translation regulatory sequence at least one fragment of tumor-associated gene and one fragment of cytokine gene. The resulting DNA vaccine contains both tumor-associated gene and cytokine gene.

The expression of tumor-associated gene and cytokine gene incorporated into the aforesaid vector may at least be controlled by one or

more mammalian expression promoters, such as CMV, PSV or LTR, or regulated by IRES. Said tumor-associated gene may be a oncogene, such as neu, met or ras, in one complete or truncated segment, for example, a fragment of N'-neu gene encoding the extracellular domain of neu protein. Said cytokine gene includes at least the IL-2, IL-4 or GM-CSF gene.

Another objective of the present invention is to provide a method of preparing a DNA vaccine containing tumor-associated gene and cytokine gene, comprising at least the following steps: designing primer sequence containing proper restriction site; using polymerase chain reaction (PCR) to amplify and isolate tumor-associated gene and cytokine gene respectively; using ligase to co-incorporate respectively the tumor-associated gene and cytokine gene into a vector having a suitable promoter or translation regulatory sequence.

The said tumor-associated gene may be located in front or behind the cytokine gene on the vector. The aforesaid co-incorporation can be achieved at least by way of: combining the tumor-associated gene and the cytokine gene into a fusion gene to be controlled by the same promoter, for instance, fusing N'-neu gene and IL-2 gene behind a CMV promoter; or having two independent genes that are controlled respectively by two separate promoters, for instance, inserting respectively N'-neu gene and IL-2 gene behind two separate promoters; or having two independent genes that are respectively controlled by a promoter and an IRES segment, for instance, inserting N'-neu gene behind CMV promoter and IL-2 gene behind IRES.

The aforesaid DNA vaccine may be carried at least by retroviral vector, adenoviral vector, adeno-associated viral vector, or liposome, or administered directly in the form of DNA. The aforesaid viral vectors offer higher transfection efficiency and better expression, but each of them has its limitation. For instance, retroviral vector can only transfect cells in division; adenoviral vector tends to induce strong immune response; and adeno-associated viral vector has limited gene capacity. Non-viral vectors described above, such as liposome is very safe, but its transfection efficiency and

expression are not as desirable as viral vectors. The proper vector for DNA vaccine of the present invention can be selected based on the actual needs. The DNA vaccine may be injected into muscle cell directly in the form of DNA on account of the fact that muscle cell will automatically ingest the DNA and express it, which is sufficient to elicit enhanced immune response despite of the relatively low expression level.

The DNA vaccine herein may be administered at least by way of subcutaneous injection, intramuscular injection, oral administration, spraying or gene gun injection.

BRIEF DESCRIPTION OF THE PREFERRED EMBODIMENTS

The preparation and efficacy of DNA vaccine in the present invention are further depicted with the illustration of embodiments.

EMBODIMENT 1 - Making a DNA vaccine by fusing N'-neu gene and mature IL-2 fragment

In this embodiment, DNA recombination technique is used to make a DNA vaccine containing N'-neu gene encoding the extracellular domain of neu protein and a mature IL-2 fragment. The preferred primer sequences for constructing the DNA vaccine of present invention, as used herein, includes the forward *Hind III*-N'-neu primer (SEQ ID NO:1), the reverse N'-neu-*Not I* primer (SEQ ID NO:2), the forward *Not I*-IL2 primer (SEQ ID NO:3) and the reverse IL-2-*xba I* primer (SEQ ID NO:4). Fig. 1 shows the preferred primer sequence for constructing a DNA vaccine according to this invention that contains the fusion of N'-neu gene and mature IL-2 fragment. The steps include designing a proper restriction site and isolating the desired N'-neu gene and mature IL-2 fragment using polymerase chain reaction, and after restriction enzyme digestion, adding ligase to first constructing N'-neu gene on a mammalian expression vector pRC/CMV, and then using restriction enzyme and ligase to insert a mature IL-2 fragment downstream of N'-neu

gene to form a DNA plasmid containing N'-neu-IL-2 fusion gene. As shown in Fig. 2, said DNA plasmid is inserted into *Escherichia coli* DH5α for mass reproduction and then extracted with Endofree Oiagen plasmid-Mega kits to complete the preparation of a DNA vaccine containing the fusion of N'-neu gene and mature IL-2 fragment.

EMBODIMENT 2 – The tumor-suppressing effect of N'-neu-IL-2 fusion DNA vaccine

In this embodiment, cohorts of mice were injected with $1*10^6$/ml MBT-2 bladder cancer cells on the back to induce tumor growth. Ten days later, N'-neu-IL-2 fusion DNA vaccine prepared according to Embodiment 1 or normal saline (as control) was administered intramuscularly the first time into the tumor site. The second and third administrations of vaccine took place on day 7 and day 14 after the first administration respectively (see Fig. 3 for flow process). The sizes of tumors measured at the time of first administration and 2-3 times each week afterwards are shown in Fig. 4. It is found that in comparison with normal saline, N'-neu-IL-2 DNA vaccine has marked tumor-suppressing effect.

EMBODIMENT 3 – The effect of N'-neu-IL-2 fusion DNA vaccine on the survival rate of mice

In this embodiment, cohorts of mice were injected with $1*10^6$/ml MBT-2 cells on the back to induce tumor growth. Ten days later, tumor approximately $25mm^3$ in size grew from the injection site, and N'-neu-IL-2 fusion DNA vaccine prepared according to Embodiment 1 was administered intramuscularly the first time into the tumor; some mice received normal saline or DNA vaccine containing only N'-neu, but not IL-2 as control groups. The second and third administrations of vaccine took place on day 7 and day 14 after the first administration respectively (see Fig. 3 for flow process). As shown in Fig. 5, all mice (37) administered with normal saline died in 56 days after being inoculated with MBT-2 cells (line a), while 8 of the 37 mice that received N'-neu DNA vaccine survived (survival rate of

22% as shown in line d), and 18 of the 37 mice that received N'-neu-IL-2 fusion DNA vaccine survived (survival rate of 49% as shown in line e). If the observation time was extended another three weeks, that is, 90 days after the inoculation of MBT-2 cells, only 4 out of 37 mice that were administered with N'-neu DNA vaccine survived (survival rate of 11%, as shown in line d), while 12 out of 37 mice that received N'-neu-IL-2 survived (survival rate of 32% as shown in line e). The results suggest that N'-neu-IL-2 fusion DNA vaccine is more effective than N'-neu DNA vaccine in slowing down the growth of tumor, and more effectively prolonging the life of mice in the long run.

EMBODIMENT 4 – Comparing the effect of N'-neu-IL-2 fusion DNA vaccine and the combination of N'-neu vaccine and IL-2 vaccine given separately on the survival rate of mice

To further demonstrate the progressive nature of the prevent invention, this embodiment compares the effect of N'-neu-IL-2 fusion DNA vaccine and the combination of N'-neu vaccine and IL-2 vaccine given separately on tumor suppression. The method of tumor cell injection and the time for administering DNA vaccines in this experiment are shown in Fig. 3. The results, as illustrated in line c and line e, show that 90 days after the mice were injected with MBT-2 cells, 12 out of 37 mice administered with N'-neu-IL-2 fusion DNA vaccine survived (survival rate of 32% as shown in line e), while only 1 out of 16 mice administered with combination of N'-neu vaccine and IL-2 vaccine given separately lived (survival rate of 6% as shown in line c), further indicating the superior effect of N'-neu-IL-2 fusion DNA vaccine.

The DNA vaccine of the present invention has been disclosed in the embodiments. However the embodiments should not be construed as a limitation on the spirit and scope of the appended claims .Those skilled in the art can easily understand that all kinds of alterations and changes can be made within the spirit and scope of the appended claims.

The DNA vaccine of the present invention containing tumor-associated gene and cytokine gene offers several advantages: (1) It can induce cellular and humoral immune responses which last for a long time; (2) the expressed antigen has a structure that approximates that expressed in human body during natural infection, thereby producing better immunization effect; (3) it can cross recognize different parts of the antigen, which helps overcome the problem of vaccine escape mutant; (4) combined immunization may be carried out by inserting combination of different antigen genes in the plasmid; (5) it has a variety of administration routes, including subcutaneous, intramuscular, oral, spray or gene gun; and (6) its preparation process is simple and cost-effective. It is also easy to mass produce, transport and preserve. It is an improvement over known cancer-treating vaccines and demonstrates better efficacy.